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Ammonia and nitrite acute toxicity in juvenile piavuçu *Leporinus macrocephalus* (Actinopterygii, Anostomidae)

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Abstract: The present study aimed to evaluate the acute toxicity of non-ionized ammonia (NH_3) and nitrite (NO_2^-) in piavuçu *Leporinus macrocephalus* juveniles. Two trials (96 h) were performed in 7 L aquaria (6 fish/aquarium), in semi-static systems, with 3 replicates per treatment. On the first trial, the juvenile piavuçu (3.0 ± 0.3 g) were exposed to 7 concentrations of non-ionized ammonia (control; 0.36; 1.85; 2.43; 2.80; 3.50 and 4.86 mg NH_3-N L^{-1}). On the second trial, fish (6.7 ± 0.4 g) were exposed to 6 different nitrite concentrations (control; 80.00; 101.00; 120.50; 138.20 and 155.60 mg NO_2^--N L^{-1}). Mortality was recorded every 24 h and the lethal concentration (LC_{50}) was calculated by the Spearman-Karber method. The estimated LC_{50} of non-ionized ammonia was 3.1, 2.9, 2.5 and 1.9 mg NH_3-N L^{-1} at 24, 48, 72 and 96 h, respectively. For nitrite, the estimated values were 130.0, 116.2, 105.8 and 94.4 mg NO_2^--N L^{-1}at 24, 48, 72 and 96 h, respectively. The estimated safe level for non-ionized ammonia was 0.19 mg NH_3-N L^{-1}, while for nitrite was 9.44 mg NO_2^--N L^{-1}.Visual clinical signs of intoxication with ammonia (hyperexcitability, hyperventilation, erratic swimming, loss of equilibrium, lethargy and coma) and nitrite (hyperventilation and lethargy) were described throughout the experiment. Based on the present results, the piavuçu proved to be a tolerant species to environmental concentrations of ammonia and nitrite. However, concentrations of these compounds should not exceed 0.19 mg NH_3-N L^{-1} and 9.44 mg NO_2^--N L^{-1}.

Keywords: nitrogen compounds, mortality, aquaculture, intensive system

Resumo: Toxicidade aguda de amônia e nitrito em juvenis de piavuçu *Leporinus macrocephalus* (Actinopterygii, Anostomidae). O presente trabalho teve como objetivo avaliar a toxicidade aguda da amônia não-ionizada (NH_3) e nitrito (NO_2^-) em juvenis de *Leporinus macrocephalus*. Dois experimentos (96 h) foram realizados em aquários de 7 L (6 peixes/aquário), em sistema semi-estático, com 3 repetições por tratamento. No primeiro experimento, os juvenis de piavuçu (3,0 ± 0,3 g) foram expostos a 7 concentrações de amônia não-ionizada (controle; 0,36; 1,85; 2,43; 2,80; 3,5 e 4,86 mg NH_3-N L^{-1}). No segundo experimento, os peixes (6,7 ± 0,4 g) foram expostos a 6 diferentes concentrações de nitrito (controle; 80; 101;120,5;138,2 e 155,5 mg NO_2^--N L^{-1}). As mortalidades foram registradas a cada 24 h e a concentração letal (CL_{50}) foi calculada pelo método Spearman–Karber. A CL_{50} estimada para amônia não-ionizada foi de 3,1; 2,9; 2,5 e 1,9 mg L^{-1} NH_3 para 24, 48, 72 e 96 h, respectivamente. Já para o nitrito, os valores estimados da CL_{50} foram 130,0; 116,2; 105,77 e 94,4 mg L^{-1} para 24, 48, 72 e 96 h, respectivamente. O nível de segurança estimado para amônia não-ionizada foi de 0.19 mg NH_3-N L^{-1}, enquanto para o nitrito este valor foi de 9.44 mg NO_2^--N L^{-1}. Sintomas visuais de intoxicação com amônia (hiperexcitabilidade, hiperventilação, natação errática, perda do equilíbrio, letargia e coma) e nitrito (hiperventilação e letargia) foram observados durante o período experimental. Com base nos presentes resultados, o piavuçu demonstrou ser uma espécie tolerante a amônia e ao nitrito, entretanto as concentrações destes compostos não devem exceder 0,19 mg L^{-1} de NH_3-N e 9,44 mg L^{-1} NO_2–N.

Palavras chave: composto nitrogenado, mortalidade, aquicultura, sistema intensivo

Introduction

In modern fish-farming, which aims to increase productivity, one of its traits is the utilization of intensive farming systems with high stocking densities. In these conditions, the farming water quality and the animal welfare may be compromised by the accumulation of nitrogenous compounds in the system (Van De Nieuwegiessen *et al.* 2008; Hosfeld *et al.* 2009; Szczepkowski *et al.* 2011).

The existence of high levels of nitrogenous wastes in the water of natural environments can be attributed to the metabolism of the animals, urban, industrial and agricultural flows and decomposition of organic wastes (Randall & Tsui 2002). In confined spaces, the increase in the concentration of these compounds in the water can be related to the high density of the animals and to the diets formulated for fish with high levels of protein (NRC 1983), and may also result from the decomposition of organic matter (Durborow *et al*., 1997). Food proteins are metabolized (Van Waarde 1983; Wicks *et al*. 2002) and after the process of deamidation the excess nitrogen is excreted through the gills (Dabrowski 1986), mainly in the form of ammonia, which represents about 80% of the nitrogenous compounds excreted by most fish species (Carter *et al.*1998; Weihrauch *et al*. 2009; Dolomatov *et al.* 2011).

The presence of ammonia in the farming environment must be considered important and monitored periodically (Roumieh *et al*. 2012), because it is the nitrogenous compound that shows the highest toxicity (Randall & Tsui 2002). Ammonia occurs in two forms in the environment and their toxicity is related to the balance among non-ionized (NH_3) and ionized (NH_4^+) forms, that depends on pH, temperature and salinity. The form NH_3 shows greater risk of toxicity due to its easiness of diffusion through membranes (Randall & Tsui 2002).

Moreover, in freshwater fish, ammonia excretion occurs by diffusion due to the NH_3 favorable concentration gradient. When the environment contains an elevated NH_3 concentration, this may cause a reduction in the excretion, promoting accumulation in the blood and tissues of fish (Wilkie & Wood 1996).

Nitrite is an intermediate compound that may occur in the water or in the soil by the processes of nitrification, denitrification and reduction of nitrate to ammonia, being dissimilatory or assimilatory (Philips *et al*. 2002). In the aquatic environment, during the nitrogen cycle, ammonia tends to be oxidized to nitrite by the action of nitrifying bacteria, especially the *Nitrossomonas* genus, which through aerobic action transform NH_4^+ into NO_2^- (Teske *et al*. 1994; Jensen 2003). In fish farms that use water recirculation system, the accumulation of high levels of nitrite may occur (Kroupova *et al*. 2005).

Nitrite can be absorbed by diffusion in the gills due to its affinity with the gill’s chloride exchanger (Cl^- / HCO_3^-) and through the intestinal epithelium of the fish, affecting multiple physiological functions (Jensen 2003; Kroupova *et al.* 2005). However, its main mechanism of toxicity is related to its ability to oxidize the iron that makes up hemoglobin to form methemoglobin, a form incapable of carrying oxygen to tissues (Hilmy *et al.* 1987; Jensen 2003; Madison & Wang 2006).

Exposure to nitrite and ammonia may cause various behavioral and physiological changes in fish that may lead to growth reduction, immune impairment and even the death of the animals (Wicks *et al*. 2002; Chew *et al*. 2006). Information on the tolerance to ammonia and nitrite estimated by lethal concentration studies are extremely important, and studies have been done in several fish species (Barbieri & Doi 2012; Devaraj *et al*. 2014; Rodrigues *et al*. 2014; Medeiros *et al*. 2015; Wang *et al*. 2015).

Among the Brazilian native species with potential for intensification of aquaculture stands out the piavuçu *Leporinus macrocephalus* (Anostomidae, Garavello & Britski 1988) (Baldisserotto & Gomes 2005). It is a species from the Paraguay River Basin, which presents rapid growth in the early stages (Garavello & Britski 1988), good performance indexes, and rusticity to handling (Feiden *et al*. 2009). It also has a great capacity of metabolizing protein and energy from plants in post-larval and later stages (Navarro *et al*. 2006; Rodrigues *et al*. 2006).

However, there is still little information about its tolerance to water quality parameters, and the toxicity of ammonia and nitrite has not been evaluated for this species. Thus, the aim of this study was to determine the acute toxicity of non-ionized ammonia and nitrite to piavuçu juvenile stages, to provide information for the establishment of desirable levels of these compounds in farming systems.

Material and methods

The piavuçu *Leporinus macrocephalus* juveniles were purchased from a commercial fish farming (Piscicultura Águas do Vale®, Mato Leitão, RS) and transported to the Continental Aquaculture Laboratory – LAC (32° 5' 11"S, 52° 13' 9"O) from the Federal University of Rio Grande (FURG). At LAC the animals stayed in quarantine (stock density - 0.23 g L^{-1}) in a 7 m^3 tank , with water recirculation through mechanical and biological filters, subject to constant aeration (dissolved oxygen >7.0 mg L^{-1}) and controlled temperature (27.04 ± 1.02 ºC). Fish were fed once a day with a commercial diet (38% of crude protein).

Two experiments were performed for the determination of the LC_{50} of ammonia (experiment 1) and nitrite (experiment 2). For experiment 1, 126 juvenile piavuçu (3.0 ± 0.3 g; stock density: 2.57 g L^{-1}) were distributed in 21 experimental units (7 L), while for experiment 2, 108 fish (6.7 ± 0.4 g; stock density: 5.74 g L^{-1}) were distributed in 18 experimental units (7 L).

Fish were exposed to seven concentrations of ammonia in the experiment 1 (control – 0.01 ± 0.01; 0.36 ± 0.04; 1.85 ± 0.25; 2.43 ± 0.35; 2.80 ± 0.36; 3.50 ± 0.44; 4.86 ± 0.33 mg NH_3-N L^{-1}) and to six concentrations of nitrite in the experiment 2 (control – 0.00 ± 0.00; 80.00 ± 3.51; 101.00 ± 4.85; 120.50 ± 5.73; 138.2 ± 3.38; 155.6 ± 3.25 mg NO_2^--N L^{-1}), in triplicate, for 96 h. The desired concentrations of NH_3-N and NO_2^--N were obtained with the addition of ammonium chloride and sodium nitrite solutions to the water, respectively, or water exchange, when necessary.

The water quality parameters, temperature and dissolved oxygen (Digital Oximeter YSI EcoSense® DO200A), pH (Digital pHmeter Hanna Instruments® HI 8424), total ammonia (Unesco 1983), non-ionized ammonia (Colt 2002), nitrite (Bendschneider & Robinson 1952) and total alkalinity (Eaton *et al*. 2005) were monitored daily. Throughout the experimental period, the water quality parameters in both trials (Table I) remained within levels considered desirable for tropical freshwater fish (Boyd & Tucker 2012). The photoperiod remained 12 h light and 12 h dark. The animals were fasted 24 h before the beginning of trials and throughout the 96 h exposure.

The mortality data were recorded every 24 h and the LC_{50} and its confidence interval (CI 95%) was estimated by the Spearman-Karber (Hamilton *et al*. 1977). The safe level was estimated from the multiplication of the LC_{50} by the application factor (0.1), according to Sprague (1971). Fish behavior was also monitored daily for 30 min (Ferreira *et al.* 2013). The fish in the control treatments served as a reference to indentify clinical signs of intoxication in those exposed to the different concentrations of non-ionized ammonia and nitrite (Absalom *et al.* 2013).

Table I. Water quality parameters (means ± standard deviation) during experimental period.

Parameters	Experiment 1	Experiment 2
Temperature (ºC)	23.38 ± 0.58	22.98 ± 0.21
pH	7.61 ± 0.06	7.65 ± 0.13
Alkalinity (mg $CaCO_3$ L^{-1})	60.57 ± 5.51	63.30 ± 1.07
Dissolved oxygen (mg L^{-1})	8.15 ± 0.68	7.78 ± 0.14

Results

The data of cumulative mortality related to each treatment throughout the experimental period are shown in tables II and III. The estimated LC_{50} for ammonia and its respective confidence intervals were 3.1 (3.1-2.9), 2.9 (2.9-2.7), 2.5 (2.6-2.3) and 1.9 (2.2-1.6) mg NH_3-N L^{-1} for 24, 48, 72 and 96 h, respectively (Table II). For nitrite, the following values were estimated 130 (140.0-122.2), 116.2 (122.3-110.5), 105.77 (111.5-100.3) and 94.4 (108.5-82.0) mg NO_2^--N L^{-1} for 24, 48, 72 and 96 h, respectively (Table III). The estimated safe level for non-ionized ammonia was 0.19 mg NH_3-N L^{-1}, while for nitrite was 9.44 mg NO_2^--N L^{-1}.

During the ammonia exposure, in all tested concentrations, except in the lowest (0.36 mg NH_3-N L^{-1}), fish presented clinical signs of intoxication, such as hyperexcitability, hyperventilation, erratic swimming, loss of equilibrium, lethargy and coma. In the nitrite exposure experiment, only hyperventilation and lethargy were observed in fish in all tested concentrations (80.00 – 155.60 mg NO_2^--N L^{-1}).

Table II. Results of cumulative mortality throughout 96h of exposure to different concentrations of non-ionized (mg NH_3-N L^{-1}) and estimated LC_{50} (mg NH_3-N L^{-1}) with confidence intervals.

Cumulative Mortality (%)								
Hours	0.01	0.36	1.85	2.43	2.80	3.50	4.86	LC_{50}
24	0	0	0	0	27.75	88.88	100	3.1 (3.2 – 2.9)
48	0	0	0	0	61.05	100	100	2.8 (2.9 – 2.7)
72	0	0	0	38.88	83.25	100	100	2.5 (2.6 – 2.3)
96	0	0	16.66	77.77	100	100	100	1.9 (2.2 – 1.6)

Table III. Results of cumulative mortality throughout 96h of exposure to different concentrations of nitrite (mg NO_2^--N L^{-1}) and estimated LC_{50} (mg NO_2^--N L^{-1}) with confidence intervals.

Cumulative Mortality (%)							
Hours	0.00	80.00	101.00	120.50	138.20	155.60	CL 50
24	0	0	0	55.55	94.44	100	130.0 (140 – 122.2)
48	0	0	16.66	77.77	94.44	100	116.2 (122.3 – 110.5)
72	0	0	33.33	88.88	100	100	105.8 (111.5 – 100.3)
96	0	33.33	55.55	88.88	100	100	94.4 0(108.5 – 82)

Discussion

The toxicity of non-ionized ammonia may be influenced by environmental parameters, such as salinity (Costa *et al*. 2008, Barbieri & Doi 2012), temperature (Barbieri & Bondioli 2015) and pH (Miron *et al*. 2008; Chezhian *et al*. 2012), and other factors like life stage and size of the animals (Gomulka *et al*. 2014; Wang *et al*. 2015), making it difficult to compare between results from different studies. However, in general, for freshwater fish, the values of LC_{50-96h} described in the literature vary from 0.16 mg L^{-1}, for more sensitive species like the trout (Thurston & Russo 1983), to 3.4 mg L^{-1} as described by Thurston *et al*. (1981) for *Pimephales promelas* (cyprinidae, Rafinesque, 1820).

When compared to LC_{50-96h} (0.27 mg L^{-1}) obtained by Copatti et al. (2015) for another species of the same genus, *L. obtusidens* (Anostomidae, Valenciennes, 1837), *L. macrocephalus* presented higher tolerance to NH_3. Studies with juvenile of other freshwater species, performed in warm waters and in similar pH conditions to this study, Miron *et al*. (2008) reported that for juvenile *Rhamdia quelen* (Pimelodidae, Quoy & Gaimard, 1824), the estimated LC_{50-96h} was 1.45 mg L^{-1}, while Evans *et al*. (2006) demonstrated that for tilapia *Oreochromis niloticus* (Cichlidae, Linnaeus, 1758) this value is 0.98 mg L^{-1}, similar to the obtained for common carp *Cyprinus carpio* (Cyprininae, Linnaeus, 1758) (0.93 mg L^{-1}) by Abbas (2006). The cardinal tetra *Paracheirodon axelrodi* (Characidae, Schultz, 1956), Amazon ornamental species, has shown higher sensitivity to non-ionized ammonia with a LC_{50-96h} calculated in 0.36 mg L^{-1} (Oliveira *et al*. 2008). The result found for juvenile piavuçu (1.9 mg L^{-1}) in the present experimental condition demonstrates that the species shows elevated tolerance to non-ionized ammonia in the water.

The nitrite toxicity for fish has been widely discussed and among the toxic effects of nitrite it must be emphasized the oxidation of hemoglobin into methemoglobin, a form unable to bind with oxygen (Urrutia & Tomasso 1987; Wuertz *et al*. 2013; Park *et al*. 2013; Saoud *et al*. 2014; Ciji *et al*. 2015). The toxicity of nitrite can be influenced by salinity, presenting an inversely proportional relationship as per some studies have shown (Atwood *et al*. 2001; Kroupova *et al*. 2005; Costa *et al*. 2008; Wuertz *et al*. 2013), which is due to the affinity that nitrite presents with the channels of chloride uptake located in the gills (Tomasso & Grosell 2005). Thus, the nitrite accumulation becomes a potential problem and requires greater attention in the farming of freshwater species such as the piavuçu.

The results of the present study demonstrated that for *L. mracocephalus* the nitrite LC_{50-96h} is 94.4 mg L^{-1}. Other freshwater species present an even higher tolerance to this compound, such as *Channa striata* (Channidae, Bloch, 1793), whit a LC_{50-96h} of 216.22 mg L^{-1} (Lefevre *et al*. 2012), *Micropterus salmoides* (Centrarchidae, Lacepède, 1802), with the reported value of 140 mg L^{-1} (Palachek & Tomasso 1984) and zebra-fish *Danio rerio* (Cyprinidae, Hamilton, 1822) (242.55 mg L^{-1}) (Doleželová *et al*. 2011). However, when compared to species such as *Oncorhynchus mykiss (*Salmonidae, Walbaum, *1792)* and *Oncorrhynchus clarkii* (Salmonidae, Richardson, 1836) (<1 mg L^{-1}) (Russo *et al*. 1974; Thurston *et al*. 1978), *Rhamdia quelen* (20.46 mg L^{-1}) (Lima *et al*. 2011), *Colossoma macropomum* (Characidae,Cuvier, 1816) (5.98 mg L^{-1}) (Costa *et al*. 2004) and *Brycon cephalus* (Characidae, Günther, 1869) (0.86 mg L^{-1}) (Avilez *et al*. 2004), it is evident that the piavuçu is a species that shows elevated tolerance to nitrite in the environment.

In the absence of studies assessing the chronic or sub-lethal effects of the exposure to a specific compound, the estimated LC_{50} provides relevant information that may be used to determine a safe level, according to what proposed Sprague (1971). The present results suggest that concentrations above 0.19 mg NH_3-N L^{-1} and 9.44 mg NO_2^--N L^{-1}, may cause deleterious effects for juvenile piavuçu and should be avoided in aquaculture farming systems. In recent years, the production in intensive systems such as recirculation aquaculture system (RAS) has been developed to increase production, animal welfare and environmental sustainability. However, lethal or sublethal NH_3 and NO_2^- levels may occur, if the biofilters are not mature or poorly designed (Martins *et al.* 2010). Thus, the determination of ammonia and nitrite safe levels provides relevant information for the proper planning and management of production systems to maintain concentrations within desirable levels for the species.

The observation of behavioral changes can be a useful and practical tool used by fish farmers to identify undesirable levels of nitrogenous compounds and fish welfare in production systems (Rodrigues *et al.* 2011; Martins *et al.* 2012). Similarly as described for other species (Knoph 1992; Benli & Koksal 2005; Xu *et al*. 2005; Hanna *et al*. 2013; Zang *et al*. 2013; Medeiros *et al.* 2015), the juvenile piavuçu exposed to ammonia showed various clinical signs, such as hyperexcitability with higher swimming activity and constant leaping, hyperventilation, erratic swimming, loss of equilibrium and lethargy. In addition, before death, fish went through a brief period of coma state, characterized by the absence of movements and response to mechanical stimuli, only remaining a reduced opercular beat (Knoph 1992).

During the exposure to nitrite it was observed that the juvenile piavuçu presented hyperventilation and reduced their swimming activity, which is according to what was described for other fish species (Williams *et al*. 1997; Lefevre *et al*. 2011; Wuertz *et al*. 2013). These behavioral changes may indicate an attempt of raising the oxygen transport by the increase of ventilation rates (Aggergaard & Jensen 2001) and of reducing its consumption by decreasing its metabolism (Espina & Alcaraz 1993), thus minimizing the effects of hypoxia due to methemoglobin formation.

The results of the present study demonstrate that piavuçu is a species that shows good resistance to ammonia and nitrite in the water. However, concentrations above 0.19 mg L^{-1} NH_3-N and 9.44 mg L^{-1} NO_2^--N must be avoided to prevent possible deleterious effects to fish welfare. Future studies must be performed to elucidate the physiological mechanisms involved in the high tolerance of this species to nitrogenous compounds, as well as the effects of exposure to sublethal levels and its possible implications over the welfare and productive performance of fish.

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